Cocoa diet modulates gut microbiota composition and improves intestinal health in Zucker diabetic rats

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Abbreviations used: AUC, area under the curve; **GM**, gut microbiota; **GTT**, Glucose tolerance test; **HOMA-IR,** homeostasis model assessment of insulin resistance; **IL-6**, interleukin-6; **MCP-1**, monocyte chemoattractant protein 1; **PCNA**, anti-proliferating cell nuclear antigen; **SCFA**, short chain fatty acids; **STZ**, streptozotocin; **T2D, type 2 diabetes; TNF-α**, tumour necrosis factor-α; **ZDF**, Zucker diabetic fatty; **ZL**, Zucker lean; **ZO-1**, Zonula occludens-1

Abstract

Cocoa supplementation improves glucose metabolism in Zucker diabetic fatty (ZDF) rats via multiple mechanisms. Furthermore, cocoa rich-diets modify the intestinal microbiota composition both in humans and rats in healthy conditions. Accordingly, we hypothesized that cocoa could interact with the gut microbiota (GM) in ZDF rats, contributing to their antidiabetic effects. Therefore, here we investigate the effect of cocoa intake on gut health and GM in ZDF diabetic rats.

Male ZDF rats were fed with standard (ZDF-C) or 10% cocoa-rich diet (ZDF-Co) during 10 weeks. Zucker Lean animals (ZL) received the standard diet. Colon tissues were obtained to determine the barrier integrity and the inflammatory status of the intestine and faeces were analysed for microbial composition, short-chain fatty acids (SCFA) and lactate levels. We found that cocoa supplementation up-regulated the levels of the tight junction protein Zonula occludens-1 (ZO-1) and the mucin glycoprotein and reduced the expression of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1) in the colon of ZDF diabetic animals. Additionally, cocoa modulated the microbial composition of the ZDF rats to values similar to those of the lean group. Importantly, cocoa treatment increased the relative abundance of acetate-producing bacteria such as *Blautia* and prevented the increase in the relative amount of lactate-producing bacteria (mainly *Enterococcus* and *Lactobacillus* genera) in ZDF diabetic animals. Accordingly, the total levels of SCFA (mainly acetate) increased significantly in the faeces of ZDF-Co diabetic rats. Finally, modified GM was closely associated with improved biochemical parameters related to glucose homeostasis and intestinal integrity and inflammation.

These findings demonstrate for the first time that cocoa intake modifies intestinal bacteria composition towards a healthier microbial profile in diabetic animals and suggest that these changes could be associated with the improved glucose homeostasis and gut health induced by cocoa in ZDF diabetic rats.

Keywords: Diabetes type 2; Cocoa flavanols; Gut microbiota; Gut barrier; Gut inflammation; Glucose homeostasis.

Highlights.

- Intestinal barrier integrity is improved in diabetic rats submitted to cocoa diet
- Cocoa diet prevents intestinal inflammation in diabetic rats
- Cocoa diet modifies gut microbiota to a healthier microbial profile in diabetic rats
- Total levels of short-chain fatty acids increases in diabetic rats fed on cocoa

1.- Introduction

Diabetes is a complex metabolic disorder characterized by hyperglycaemia resulting from defects in insulin secretion and insulin action (ADA, 2017). At present, prevalence of type 2 diabetes (T2D) is reaching epidemic proportions, becoming a serious threat to public health worldwide mainly due to the associated complications (WHO, 2016). The growing prevalence of T2D is positively related to harmful lifestyles, in particular the reduced levels of physical activity and increasingly unhealthy eating habits, indicating that diet plays a crucial role in the onset and progression of T2D (WHO, 2016). More importantly, an increasing body of evidence suggests that certain dietary compounds may attenuate the risk of T2D by their ability to modulate gut microbiota (GM) composition (Nie et al., 2019).

The GM comprises a complex community of bacteria that colonizes the surface and the lumen of the gastrointestinal tract. Microbiota transform food components and produce a wide range of derived metabolite that impact host's physiology and health in many ways, from the maintenance of intestinal homeostasis to energy metabolism (Van Treuren & Dodd, 2020). Indeed, many disease states have been associated with alterations in microbiota composition and consequently on their functionality, indicating that GM could be involved in the development of numerous pathologies (Danneskiold-Samsoe et al., 2019). In particular, microbiota function has been identified as a relevant and potentially modifiable factor that contributes to the development of metabolic diseases, including T2D (Li, Watanabe & Kimura, 2017). Therefore, interventions targeting GM are emerging as promising effective strategies for the prevention and management of T2D.

Polyphenols are a large group of phytochemical compounds that have attracted much interest due to their beneficial properties. Accumulating evidences suggest that dietary polyphenols may interact with GM (Nash et al., 2018; Tomás-Barberán & Espín, 2019). Bioavailability of dietary

polyphenols in the digestive tract is highly variable. Aglycones, monomeric and dimeric structures can be absorbed in the small intestine. However, most of polymeric structures reach the colon intact where they are metabolized by GM producing small microbial derived metabolites which are absorbed more efficiently and therefore may contribute to the beneficial health effects of polyphenols (González-Sarrías, Espín, & Tomás-Barberán, 2017). In addition, these natural compounds modulate the composition and function of GM exhibiting prebiotic effects and antimicrobial action against pathogenic intestinal microbiota (Marchesi et al., 2016; Singh et al., 2019). In this way, polyphenols can influence the bacterial production of fermented or degraded metabolites (short chain fatty acids – SCFA-) which can modulate multiple physiological pathways in several tissues, affecting gut health, glycaemic control, lipids profile and insulin resistance (Morrison, & Preston, 2016). Therefore, a more complete understanding of this bidirectional interaction between polyphenols and GM should help to explain the beneficial health effects of these natural compounds.

Cocoa is considered a rich source of dietary polyphenols, mainly flavanols such as epicatechin and procyanidins. Cocoa flavanols can exert antidiabetic effects via multiple mechanisms, including antioxidant and anti-inflammatory effects, as well as by increasing insulin secretion and insulin action (Martín, Goya, & Ramos, 2016). In addition, a cocoa rich diet has been described as able to modify the intestinal microbiota composition in healthy rats (Massot-Cladera et al., 2012; Massot-Cladera et al., 2014), pigs (Jang et al., 2016; Magistrelli et al., 2016) and humans (Tzounis et al., 2011). Therefore, it is probable that interaction of cocoa components with GM actively contributes to the antidiabetic effects of cocoa. However, to date, the influence of cocoa feeding on intestinal health and on the composition of GM in diabetes remains to be considered. Accordingly, the aim of the present study was to investigate whether cocoa supplementation modulate intestinal dysbiosis induced by diabetes in an *in vivo* model of T2D, using Zucker diabetic fatty (ZDF) rats. To this end, the effect of a cocoa rich-

diet on glucose homeostasis and biomarkers of gut health in diabetic ZDF rats was evaluated. In addition, the compositional changes in GM and SCFAs induced by cocoa in diabetic animals were also determined.

2. Material and Methods

2.1. Diets, animals and experimental design

Diets were prepared from an AIN-93G formulation (Panlab S.L., Barcelona, Spain). Cocoa rich-diet (10%) was produced by adding 100 g/Kg of natural Forastero cocoa powder (a kind gift from Idilia Foods, Barcelona, Spain) to AIN-93G diet. It contains epicatechin (382 mg/100 g), catechin (115 mg/100 g) and procyanidins (167 mg/100 g) and non-flavonoid compounds such as theobromine (742mg/100 g). The resulting cocoa diet was isoenergetic and its composition is given in Table 1.

Male Zucker diabetic fatty (ZDF) rats (n=16) and their Zucker lean controls (ZL) (n=6) were obtained from Charles River Laboratories (L'arbresle, France) at 9 weeks of age. Animals were placed under standard controlled conditions (21 ºC ± 1 ºC; 12 h day/night cycle). After one week of acclimation, ZDF diabetic rats were randomly divided into two groups of eight animals that received the standard AIN-93G diet (ZDF-C) or the same control diet supplemented with 10 % of cocoa (ZDF-Co) for 10 weeks. The lean Zucker rats (ZL) received the standard AIN-93G diet. During the experiment, food and water were available *ad libitum.* Food intake was monitored daily and animal weight and glycaemia was weekly followed. Animals were treated according to the European (2010/63/EU) and Spanish (RD 53/2013) legislation on Care and Use of Experimental Animals and the experiments were approved by the Ethics Committee from Comunidad de Madrid (PROEX 304/15).

2.2. Biochemical determinations

At 20 weeks of age, animals were fasted overnight and were scarified by exsanguination under anaesthesia ketamine/xylazine (80 mg/8 mg Kg^{-1}, i.p.). Blood samples were collected for biochemical analysis. Glucose was determined using an Accounted Glucose Analyzer (LifeScan España, Madrid, Spain) and insulin and glycosylated haemoglobin (HbA1c) were quantified by ELISA kits (Rat Insulin,

Mercodia, Uppsala, Sweden; HbA1c Kit Spinreact, BioAnalitica, Madrid, Spain). Fasting plasma concentrations of both glucose and insulin were used to calculate indices of insulin resistance [homeostasis model assessment (HOMA)-IR] and secretion (HOMA-B) using the following formulae: HOMA-IR = fasting insulin (mU/ml) X fasting glucose (mM)/22.5; HOMA-B = 20 X fasting insulin (mU/ml)/[fasting glucose (mM)-3.5]. Triacylglycerols (TG), HDL-Cho and LDL-Cho were determined in serum by kits (BioSystems, Madrid, Spain) as described elsewhere (Álvarez-Cilleros et al., 2019). Tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were quantified in serum samples by specific rat TNF-alpha Quantikine ELISA Kit (RTA00, R&D System, USA) and IL-6 DuoSet ELISA Kit (DY506, R&D System, USA) according to the manufacturer's instruction.

2.3. Glucose tolerance test (GTT)

GTTs were performed one week before the end of the study. Briefly, an overload of glucose (2g/Kg body weight) (Sigma Chemical, Madrid, Spain) was ip administered in animals subjected to overnight fasting. Blood samples were collected from the tail vein at five different time points (0, 30, 60, 90, 120 and 180 min) and glucose levels were measured using an Accounted Glucose Analyzer (LifeScan España, Madrid, Spain). The integrated glucose response (area under the curve, AUC) over a period of 180 min after glucose overload was also calculated.

2.4. Histologic and immuno-histochemical analysis

Rats were sacrificed at 20 weeks of age and the entire colon was resected and cleaned with PBS. Sections (2 cm) from the most distal portion of the colon were routinely processed and paraffin embedded for histological and immune-histochemical analyses. Sections were cut, stained with haematoxylin–eosin (H&E) or periodic-acid-Schiff (PAS) according to the manufacturer's instructions.

Images were obtained under light microscopy (Leica DM LB2) and a digital Leica DFC 320 camera (Leica, Madrid, Spain) and quantified with ImageJ software (Fiji image J; 1.52i, NIH, USA). The crypt depth was measured from H&E slices and was determined as number of cells per hemi-crypt. Only crypts with an open longitudinal crypt axis were analysed. The tissue expression level of the neutral mucin glycoprotein was determined by means of PAS and it was calculated as the number of PAS positive cells per crypt.

For the immuno-histochemical staining, antibodies against monoclonal anti-proliferating cell nuclear antigen (PCNA; PC-10) (Lab Vision Corporation and Bionova- Científica SL), Cyclin E (sc-247, Santa Cruz Biotechnology), p21 (sc-6246, Santa Cruz Biotechnology), Zonula occludens-1 **(**ZO-1) (sc-10804, Santa Cruz Biotechnology)**,** tumour necrosis factor-α (TNF-α) (sc-133192, Santa Cruz Biotechnology), interleukin-6 (IL-6) (sc-57315, Santa Cruz Biotechnology), monocyte chemoattractant protein 1 (MCP-1) (sc-52701, Santa Cruz Biotechnology) and CD45 (ab-10558, Abcam) were used. After deparaffinization and endogenous peroxidase quenching, serial colonic sections were incubated with the primary antibodies overnight at 4ºC. Secondary antibodies were used to detect primary antibodies, followed by streptavidin-tagged horseradish peroxidase and visualized by 3,3′-diaminobenzidine (DAB) substrate (Sigma Chemical, Madrid Spain). The sections were counterstained with Harris's haematoxylin, dehydrated and mounted. Brown colour indicates specific protein immunostaining and light blue colour indicates nuclear haematoxylin staining. Positive and negative controls were used during the optimization of the methods.

At least 20 perpendicular well-oriented crypts were examined in each animal under light microscopy at x400 magnification. The proliferative labelling index (LI) (%) was calculated as the number of positive nuclei × 100/total number of cells per crypt column height. ZO-1, TNF-α, IL-6,

MCP-1 and CD45 protein expression level was evaluated as percentage of the stained area to the total area per crypt by using the colour deconvolution plug-in from ImageJ v1.52j software.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

Apoptotic colonic epithelial cells were labelled *in situ* by identifying DNA fragmentation on paraffin embedded sections using the terminal deoxynucleotidyl transferase UTP nick end labelling (TUNEL) assay. After deparaffinization and rehydration tissue sections were permeabilized with proteinase K (20 μg/mL) for 15 min at 37°C, and then treated with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity. After sections were incubated with equilibration buffer for 10 min, followed by immediate application of TdT-enzyme working for 1 h at 37ºC. Slices were incubated with peroxidase conjugated streptavidin and subsequent staining with DAB and counterstaining with methyl green. The apoptotic index represents the proportion of cells undergoing apoptosis within a crypt column (x400) and was calculated as the ratio of TUNEL-positive cells to the total number of cells counted within 50 full-length well orientated crypts.

2.6. Faecal samples.

Fresh faecal samples were collected at the end of the intervention period, early in the morning, by abdominal massage in sterilized tubes and immediately frozen at –80ºC for future analyses.

2.7. DNA extraction, and 16S gene PCR amplification. Illumina Mi-Seq sequencing.

DNA was extracted from faecal samples using G-spin columns (INTRON Biotechnology). DNA concentration was determined using Quant-IT PicoGreen reagent (ThermoFisher Scientific, Inc., Waltham, MA, USA) and around 3 ng were used to amplify the V3-V4 region of 16S rRNA gene

(Caporaso et al., 2011). PCR products (approx. 450 bp) included extension tails, which allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR. Individual amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and a pool of samples was made in equimolar amounts. The pool was further cleaned, quantified and the exact concentration estimated by real time PCR (Kapa Biosystems). Finally, DNA samples were sequenced on an Illumina MiSeq instrument with 2 x 300 paired-end read sequencing at the Unidad de Genómica (Parque Científico de Madrid, Spain).

We used the BIPES pipeline to process the raw sequences (Zhou et al., 2011) and we performed UCHIME (implemented in USEARCH, version 6.1) to screen out and remove chimeras in the *de novo* mode (Edgar, & Flyvbjerg, 2015). In each sample between 90,000 and 220,000 sequences were identified. All subsequent analyses were performed using 16S Metagenomics (Version: 1.0.1.0) from Illumina. The sequences were then clustered to an operational taxonomic unit (OTU) using USEARCH with default parameters (USERACH61). The threshold distance was set to 0.03. Hence, when the similarity between two 16S rRNA sequences was 97%, the sequences were classified as the same OTU. QIIME-based alignments of representative sequences were performed using PyNAST, and the Greengenes 13_8 database was used as the template file. The Ribosome Database project (RDP) algorithm was applied to classify the representative sequences into specific taxa using the default database (Edgar, & Flyvbjerg, 2015). The Taxonomy Database (National Center for Biotechnology Information) was used for classification and nomenclature. Bacteria were classified based on the SCFA end-product as previously described (Wang, Garrity, Tiedje, & Cole, 2007).

2.8. Measure of SCFAs and lactate in faeces

Faecal samples were weighed and suspended in 1 mL of water with 0.5% phosphoric acid per 0.1 g of sample and frozen at −20ºC immediately after collection. Once thawed, the faecal suspensions were homogenized with a vortex for about 2 min and centrifuged for 10 min at 17949 g. Aliquots of 400 µL of supernatans were diluted with 100 µL of 4-methyl valeric acid used as internal standard at a final concentration of 788 µM and then the suspension was extracted with 1 mL of n-butanol for 21 min and centrifuged for 105 min at 16000 g. A stock solution containing the mixture of standards (WSFA-2; Sigma-Aldrich, Madrid, Spain) was treated as samples and diluted to obtain a calibration curve ranging from 2 to 10000 µM. Internal standard was also added to the mixture of standards.

The analytes (2 µL) were injected in the splitless mode into an Agilent 7890A gas chromatography (GC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with a 5975C mass spectrometer (MS) detector and an Agilent DB-WAXtr column (100% polyethilen glycol, 60 m, 0.325 mm, 0.250 µm). Helium was used as a carrier gas at 1.5 mL/min. The column temperature was initially 50ºC, then increased to 150ºC at 15ºC/min, to 200ºC at 5ºC/min, and finally to 240ºC at 15ºC/min and kept at this temperature for 20 min (total time 41.3 min). The MS was tuned during all experiments; the signal acquisition for quantification was done in the single-ion monitoring (SIM) mode. The temperature of the ionization source and the quadrupole were 230ºC and 150ºC, respectively. The electron-impact ionization energy was 70 eV. Concentrations of acetate, propionate and butyrate were expressed as µM/g of sample.

For lactate determination faecal samples were suspended in MilliQ water. After homogenization with an ultrasonic liquid processor Vibra-Cell CV18 (Sonics & Materials, Connecticut, Unites States), faecal suspensions were centrifuged at 1000 g for 5 min. The aqueous phase was filtered through a 0.2 µm cellulose acetate syringe filter (VWR International, Barcelona, Spain). After filtration, lactate was measured with an Advanced Compact Ion Chromatographic instrument IC867 (Metrohm AG, Herisau,

Switzerland). Sodium L-lactate (Sigma-Aldrich, Madrid, Spain) was used as standard. Concentrations of lactate were expressed as µM/g of sample.

2.9. Statistical Analysis

Data from biochemical and immune-histochemical parameters, relative abundances of taxa and SCFA levels were tested for normality and homogeneity of variances by the D'Agostino and Pearson and Levene tests, respectively; for multiple comparisons, one-way ANOVA was followed by a Tukey test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A GraphPad Prism version 7.00 (GraphPad software, Inc., La Jolla, California) was used.

Shannon, Chao and Simpsons indexes were calculated to analyse α-diversity using QIIME. Reads in each OTU were normalized to total reads in each sample. Unsupervised classification studies with Principal Components Analysis (PCA) were carried out to analyse the differences between groups. Relationship strength between parameters was assessed using the two tailed Pearson's correlation test. The correlation was considered significant only when the absolute value of Pearson's correlation coefficient r was > 0.5.

3. Results

3.1. Physiological parameters

At the beginning of the study, ZDF animals showed marked increases in body weight as compared to ZL rats (210.0 ± 9,0 vs 284.4 ± 10.6 g, respectively; $P < 0.05$). However, fasted glycaemia was not significantly different between ZL and ZDF animals (84.6 ± 6.0 vs 102.2 ± 13.1 mg/dL, respectively; $p < 0.05$), which indicates that at this time point animals were at a pre-diabetic stage. Then, ZDF rats were randomly assorted to ZDF-C or ZDF-Co groups. The administration of cocoa for 10 weeks reduced body weight in diabetic ZDF rats while daily food intake remained constant (Figures 1A and 1B). Moreover, the increase in glycaemia, insulinaemia and HbA1c that is characteristic of ZDF rats was significantly reduced in those feed with cocoa diet (ZDF-Co) (Figures 1C-1D). Likewise, there was a significant reduction in insulin resistance state (HOMA-IR) and a significant increase in beta-cell function (HOMA-B) in ZDF-Co rats as compared to ZDF (Fig. 1D). Finally, the glucose tolerance test showed that diabetic ZDF-Co rats were less intolerant than ZDF-C rats and, consequently, their AUC value was significantly reduced by the cocoa rich diet (Figure 1G). In contrast, HDL-Cho, LDL-Cho and TG levels were significantly elevated in both diabetic ZDF groups in comparison to the ZL group and cocoa diet only was able to partly reduce the levels of LDL-Cho in ZDF animals (Figure 1H). Altogether, these results indicated that cocoa intake was able to improve glycemic control but not lipid profile in diabetic and obese ZDF rats.

3.2. Intestinal integrity and inflammation

Next, we investigated the effect of cocoa treatment in the intestinal barrier integrity and colonic inflammation in diabetic rats. Morphological modifications at a scale larger than crypts were not observed. As shown in Figure 2A, diabetic ZDF-C rats revealed a similar crypt depth to the ZL lean

group whereas rats fed with cocoa presented a significantly larger crypt depth. Accordingly, colonocyte proliferation and apoptosis (Figures 2B and 2C) were similar in diabetic and lean animals while both processes were significantly increased in ZDF-Co animals, indicating that cocoa intake induced a faster renewal rate of the colonic epithelium. To deepen the mechanism by which cocoa intake increases the colon mucosa and its renewal, we also evaluated the expression patterns of cell cycle proteins such as cyclin E, a late G1 phase cyclin and CDK p21 inhibitors as indicator of cell proliferation activity in colonic mucosa. ZDF-Co rats showed increases in the cytosolic and diffuse distribution of positive nuclei expression of cyclin E, compared to ZL and ZDF-C groups. The high levels of cyclins E induced by cocoa might be explained, at least in part, by a coordinate decrease in p21, reducing their association with cyclins and finally aiding the progression of cell cycle (Supplementary Figure 1). Likewise, levels of the mucin glycoprotein, a crucial component of the mucus layer expressed in goblet cells, were also slightly but significantly increased in the diabetic animals fed with cocoa (Figure 2D). In addition, control diabetic animals showed a significant decrease in the levels of one of the main tight junction proteins Zonula occludens-1 (ZO-1) in comparison with control lean group; interestingly, diabetic animals fed with cocoa not only prevented the ZO-1 decrease induced by diabetes but also showed ZO-1 levels significantly higher than those of the lean group (Figure 2E). Finally, the expression of pro-inflammatory cytokines (TNFα, IL-6, and MCP-1) and the levels of CD45 (marker of immune cell infiltration) were significantly increased in the colonic mucosa of diabetic rats and they were partly prevented in those fed with cocoa (Figure 3A-D). The circulating levels of the cytokines IL-6 and TNF-α in serum were not significantly different among ZL, ZDF-C and ZDF-Co rats (Figure 3E). Overall, these results indicate that cocoa diet to a large degree greatly maintains the intestinal integrity and generally reduced intestinal inflammation in diabetic rats.

3.3. Bacterial diversity and taxa composition

To analyse the effect of diabetes on gut microbial composition and the influence of a cocoa rich diet, we performed a metagenomic DNA sequencing of bacterial 16S rRNA gene regions V3-V4 of faecal samples. The total number of species identified was higher in ZDF-C rats when compared with ZL or ZDF-Co rats (Figure 4A). Likewise, Shannon, Simpsons and Chao indexes representing the richness and evenness of species diversity within each sample (α-diversity), were markedly augmented in control diabetic rats (Figures 4B-D). We also performed a bi-dimensional PCA of the bacterial community, which measures microorganism diversity between samples (β-diversity) in an unsupervised manner. The analysis revealed distinct clustering in each group (Figure 4E).

Microbiota in diabetic animals (ZDF-C) were characterized by a significant increase in the relative abundance of *Proteobacteria* (3.6 fold increase), *Tenericutes* (2.8 fold increase) and *Actinobacteria* (2.6 fold increase) phyla and a reduction of *Verrucomicrobia* phylum (by 76.9%) when compared with ZL non-diabetic animals (Figure 4F). Notably, with the exception of *Verrucomicrobia* phylum, the microbial changes induced by diabetes were totally prevented in animals that were fed with cocoa. In addition, cocoa significantly increased the relative percentages of *Firmicutes* (1.4 fold increase) and *Deferribacteres* phyla (9.3 fold increase) and decreased the relative abundance of *Cyanobacteries* phylum (by 74.9%) when compared to the ZDF group. Overall, colonic microbiota was dominated by the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Verrumicrobia* phylum which accounted for over 96.8%, 96.7% and 95.8% of total bacteria in ZL, ZDF-C and ZDF-Co groups, respectively. Interestingly, the most important and recognized biomarker of dysbiosis, the *Firmicutes/Bacteroidetes* (F/B) ratio, was significantly increased in both ZDF-C and ZDF-Co rats (Figure 4G).

At the family level, twenty families were identified with a relative abundance greater than 1% (Figure 5A). Eleven of these families significantly modified their relative abundance with diabetes or cocoa (Figure 5B). In particular, in ZDF-C diabetic animals, the relative abundance of *Enterobacteriaceae* (a family of the *Proteobacteria* phylum) and of *Enterococcaceae* and *Lactobacillaceae* (families of the *Firmicutes* phylum) increased whereas the abundance of *Ruminococcaceae* (of the *Firmicutes* phylum) and *Verrucomicrobiaceae* (of the *Verrucomicrobia* phylum) decreased. Interestingly, except for the *Verrucomicrobiaceae* family, cocoa diet significantly prevented all these microbial changes induced by diabetes. Moreover, cocoa supplementation increased the abundance of *Flavobacteraceae*, *Prevotellaceae* and *Sphingobacteriaceae* (families from *Bacteroidetes* phylum) and *Lachnospiraceae* (*Firmicutes* phylum) in diabetic animals.

Figure 6 shows the changes found at genus and species level. Twenty one genera were identified with abundance greater than one per cent and eleven of these were significantly modified by either diabetes or the cocoa diet. The major differences found in diabetic ZDF-C animals were observed in the genera *Escherichia* (mainly *E. alberti* species), *Tepidibacter*, *Lactobacillus* (with *L. antri*, *L. hayakitensis* and *L. johmsonii* as the most prevalent species) and *Enterococcus* (mainly *E. lactis* specie) that where significantly higher compared with non-diabetic ZL.Conversely, *Faecalibacterium* and *Oscillospira* genera were significantly lower in the ZDF-C group. Once again, cocoa diet was able to prevent all these intestinal microbiota changes in diabetic animals.However, both ZDF-C and ZDF-Co showed significantly decreased levels of *Akkermansia* genus when compared with ZL group, a finding that was also noticeable at the species levels (*A. Muciniphila*). Finally, cocoa treatment induced *Blautia* (mainly *B. hansenii* and *B. wexleare* species) and *Flavobacterium* and reduced *Parabacteroides* (mainly *P. goldsteinii* and *P. distasonis*) and *Sutterella* genera in diabetic animals.

3.4. SCFA- and lactate-producing bacteria and levels of lactate and SCFA in faeces

Next, we analysed the changes in the relative abundance of lactate- and SCFA-producing bacteria (Figures 7A and 7C-F) as well as the lactate and SCFA levels in faeces (Figure 7B). The relative abundance of butyrate-producing bacteria was essentially unchanged in all groups (Figure 7A). However, we found that cocoa feeding significantly increased the relative abundance of acetate-producing bacteria (mainly due to an induction in *Blautia* genus) (Figure 7C) that was accompanied by higher levels of acetate in faeces (Figure 7A). On the other hand, diabetic animals showed a significant increase in the amount of lactate-producing bacteria that was driven primarily by changes in *Enterococcus* and *Lactobacillus* genera (Figure 7E) and a significant reduction in the relative amount of propionate-producing bacteria that was reproduced for *Akkermansia* genus (Figure 7F). Accordingly, the levels of lactate were significantly higher in the faeces of diabetic animals while the levels of propionate were significantly lower. Notably, cocoa treatment partly prevented the increase in lactate-producing bacteria and as a result the levels of lactate and propionate were ower than those found in diabetic animals.

3.5. Correlation of gut microbiota and disease biomarkers

Finally, to further explore the relationship between the significantly altered genera in the microbiota of diabetic animals and clinical parameters related to glucose and lipid metabolism as well as intestinal integrity and inflammation, we performed a Person correlation analysis (Figure 8). A strong positive association was found between the relative abundance of *Enterococcus*, *Escherichia*, *Lactobacillus* and *Tepidibacter* genera and the increase in body weight and in several biomarkers of glucose homeostasis and inflammation. However, the genera *Oscillospira* and *Akkermansia* showed

significantly negative correlations with all these parameters. Likewise, *Fecalibacterium* showed significantly negative correlations with increase in body weight, insulinaemia, HbA1c, LDL, TG, IL-6, CD45and MCP-1. Finally, the presence of *Flavobacterium* was also negatively associated with, glycaemia, TNF-α and CD45. On the other hand, increased ZO-1 levels were positively associated with *Blautia* and *Flavobacterium* and negatively linked with *Enterococcus*, *Escherichia*, *Parabacteroides*, *Sutterella* and *Tepidibacter*.

4. Discussion

In the present study, we show for the first time that cocoa intake modifies the intestinal bacteria composition in ZDF diabetic animals towards a healthier microbial profile. Interestingly, some of the gut microbiota modifications induced by cocoa are closely associated with improved glucose homeostasis and gut health, suggesting that the beneficial effects of cocoa in diabetes could be mediated, at least in part, by modulation of the microbiota.

Early reports have revealed the potential anti-diabetic properties of cocoa both *in vivo* and *in vitro* (Martin, Goya, & Ramos, 2016). In agreement with this, here we show that cocoa supplementation partially alleviated glucose and lipid control. More importantly, in this study we found that dietary cocoa was able to improve the structure and the barrier integrity of the colon mucosa in diabetic rats. The expression of the intestinal tight junction protein ZO-1 and the intestinal mucus levels are extremely important in maintaining intestinal barrier function (Dhar & McAuley, 2019; Andrade et al., 2015). Accordingly, in the present study, ZO-1 and mucin levels were significantly reduced in the colon of control ZDF rats. However, diabetic animals fed on cocoa showed significantly higher expression of mucin and ZO-1 than those of the lean group. These results suggest that cocoa diet may improve the barrier function and integrity of diabetic animals through enhancing the protein expressions of mucin and ZO-1. Supporting this it has been shown that the intake of *Salvia miltiorrhiza*, a natural source of phenolic acids, ameliorates the damaged barrier function of diabetic mice through enhancing the expressions of tight junction proteins decreased by streptozotocin (STZ) (Gu et al., 2017).Likewise, it has been proved that long term intake of anthocyanims promoted intestinal integrity in healthy mouse (Peng et al, 2019).Notably, the tight junction of intestinal mucosal cells prevents excessive entrance of endotoxins and other noxious agents into the circulation system and therefore attenuates the activation of local and systemic inflammatory responses (Balakumar et al.,

2018). Accordingly, diabetic animals showed a significantly increase in inflammatory cytokines such as IL-6, TNFα and MCP-1 as well as in the levels of CD45 (marker of immune cell infiltration) in the colonic mucosa that were significantly reduced by cocoa intake. Although the levels of LPS coud not be assayed in the plasma of these animals, it could be highlighted that no significant differences in the levels of IL-6 and TNFα were found in plasma, which is consistent with previous data in ZDF rats at this age and fed on standard diet (Morales-Cano et al., 2019). Therefore, it could be suggested that the altered intestinal barrier function we described in ZDF rats is not sufficiently damaged to cause an overt metabolic endotoxemia. Overall, here we show that cocoa intake has the potential to improve gut barrier integrity in diabetic animals and to reduce colon inflammation emerging as an additional tool to ameliorate diabetes. Although the precise molecular mechanism behind this protective effect is still unclear, we hypothesize that it could be related in part to the potential changes induced by cocoa in intestinal bacteria of diabetic animals due to its prebiotic activity (Singh et al., 2019).

Metabolic disorders such as diabetes have been associated with altered microbiota composition (Li, Watanabe & Kimura, 2017). In this study, the composition of the GM in diabetic rats (ZDF-C) was significantly different compared with lean animals (ZL). At the phylum level, the main changes observed in diabetic animals were an increase in *Proteobacteria* and a decrease in *Verrucomicrobia* phyla whereas no significant differences were found in the relative abundance of *Firmicutes* and *Bacteroidetes*. Interestingly, microbiota in diabetic animals treated with cocoa differed between the diabetic control and the lean groups, suggesting that cocoa may have specific effects on the microbial community of diabetic animals. The reduction in *Proteobacteria* abundance in the ZDF-Co group can be attributed to the potential of cocoa polyphenols to modulate intestinal microbiota, These results are in concordance with those reported by Araujo et al. (Araujo et al., 2019), who observed a decrease in the abundance of the phyla Proteobacteria in the faeces of obese rats fed with an ethanolic extract of

bacupari rich in phenol derivatives.. However, cocoa supplementation failed to correct the increase in the *Firmicutes*/*Bacteroidetes* (F/B) ratio that is characteristic of murine genetic obese models (Vallianou et al., 2019). It has been recently suggested that the regulation of microbiota by polyphenols could be independent of the decrease in the F/B ratio (Yang et al., 2019). More importantly, a systematic review has revealed that this relationship between F/B ratio and obesity is not always consistent, suggesting changes in specific microbiota as main responsible for metabolic outcomes (Sze, & Schloss, 2016). According to that, we found that cocoa intake modified some key bacterial groups which may be related to the beneficial improvement on the glucose homeostasis and gut health induced in diabetic animals.

The relative abundance of *Proteobacteria* in diabetic ZDF-C animals was partly ascribed to a significant increase in bacteria that belong to *Escherichia* genus (*Enterobacteriaceae* family) that are widely known to cause intestinal pathologies in humans and animals (Shin, Whon, & Bae, 2016; Allen-Vercoe & Jobin, 2014). Likewise, although the absolute abundance of the *Firmicutes* phylum was unchanged in diabetic rats, certain genera were significantly modified when compared with the control lean group. Particularly, *Enterococcus and Lactobacillus* genera were increased in diabetic animals whereas *Oscillospora* genus was decreased. *Enterococcus* is a bacterial group which includes potential pathogens that have been associated with gut dysfunction and inflammatory diseases (Lo Presti et al., 2019). In contrast, *Lactobacillus* is classically considered a beneficial group of bacteria for their favourable effects on host metabolism; however, recent studies have indicated that the increase of this genus might be related to obesity and inflammatory conditions (Zeng et al., 2013; Ge et al., 2018), which is in concordance with our findings in diabetic animals. Similarly, *Oscillospira* genus is associated with anti-inflammatory effects in the gut and it has been reported to be less abundant in T2D and obese patients (Del Chieirico et al., 2017; Liu et al., 2018). Interestingly, cocoa diet strongly

prevented all these harmful changes observed in the gut microbiota of diabetic animals. These findings are in agreement with previous studies showing that quercetin and resveratrol attenuate serum inflammatory cytokines and improve glucose metabolism in high-fat diet-fed rats by modulating bacterial species associated with diseases and inflammation (Zhao et al., 2017). In the same way, modifications induced by cocoa intake on the GM of diabetic animals may play a key role in the beneficial effects on glucose metabolism and gut health.

It is interesting to note that the abundance of *Akkermansia* genus (phylum *Verrucomicrobia*) is low during obesity and diabetes (Vallianou et al., 2019). In addition, it has been demonstrated that polyphenol treatment induces the expression of *A. muciniphila* which correlated with improved body weight and glucose tolerance (Anhê et al., 2017). However, we found that cocoa supplementation failed to restore the reduced abundance of *A. muciniphyla* in diabetic animals even though glucose metabolism and gut health were significantly improved. Similar results have been reported in obese mice treated with resveratrol (Sung et al., 2017) or with polyphenol-containing extracts from cinnamon bark and grape pomace (Van Hul et al., 2018) showing improved glucose tolerance with reduced abundance of *Akkermansia*. As suggested by the authors (Van Hul et al., 2018), it is possible that polyphenols have varied prebiotic potential for *A. muciniphila,* In addition, several study design differences (origin of polyphenols, type of diet, animal model, age or pathological status) may also contribute to explain the divergence in results regarding the gut microbiota composition. Thus, further studies are required to explore the role of this bacterial group in type 2 diabetes.

Microbiota modulate the production of SCFA (mainly acetate, butyrate and propionate) which can modify the concentrations of several gut peptides involved in glucose metabolism, gut barrier function and energy homeostasis (Parada et al., 2019). Accordingly, cocoa as a prebiotic food can positively affect the growth of beneficial bacterial species (Singh et al., 2019). In particular, it has been

shown that a cocoa rich-diet promotes the growth of butyrogenic-type bacteria such as *Roseburia* in pigs (Solano-Aguilar et al., 2018). However, we found that the levels of butyrate-producing bacteria were essentially unchanged amongs all experimental groups while the abundance of acetate producing bacteria was significantly increased in cocoa fed rats. In addition, cocoa feeding increased the relative abundance of *Blautia*, a bacterial group that has been negatively correlated with obesity and T2D (Rondanelli, et al., 2015; Inoue et al., 2017). This different prebiotic effect could be attributed to significant modifications in the gut environment during the diabetic milieu which lead to the dissimilar composition and distribution of the intestinal microbiota in healthy compared to diabetic animals.

Acetate can improve gut barrier function either by stimulating goblet cell differentiation or by the reinforcement tight junctions of epithelial cells (Morrison & Preston, 2016). In this regard, it has been recently shown, in an animal model of intestinal inflammation that the supplementation with polyphenols from grape peel significantly increased the production of SCFA (mainly acetate and butyrate) and the colonic protein levels of ZO-1 (Maurer et al., 2019). Similarly, in this study, the relative abundance of *Blautia* was directly associated with increased levels of ZO-1 and mucin. Moreover, cocoa diet also decreased the abundance of lactate producing bacteria (mainly *Lactobacillus* and *Enterococcus*) and thus the level of lactate (precursor of SCFAs) in faeces. Interestingly, correlation analysis showed that both genera were positively associated with body weight and biochemical parameters related to glucose homeostasis and intestinal integrity and inflammation. Increased lactate levels have been observed in both humans and animal models of T2D and obesity and have been also associated with inflammation (Wu et al., 2016; Nishitsuji et al, 2017). However, microbial produced lactate is generally converted into propionate or butyrate by a subset of lactate-utilizing bacteria and it is unclear whether bacterial derived lactate contributes to the high levels of plasma lactate in diabetics. Additionally, it should be taken into account that gut microbiota produces

many other classes of metabolites such as SCFAs, bile acids and amino acid derivatives that may also have essential signaling functions (Van Treuren & Dodd, 2019). Altogether, here we show that cocoa could modulate GM and SCFAs production, contributing to the recovery of colon barrier function, attenuating inflammation and eventually improving glucose metabolism. Therefore, these results suggest that the modulation of GM might be one of the mechanisms involved in the antidiabetic effects of cocoa. Although we do not know whether the changes observed in fecal SCFAs concentration in both ZDF and ZDF-Co reflect similar variations in circulating SCFAs, it is reasonable to hypothesize that potential changes in systemic SCFAs levels could also contribute to the observed phenotypes of the animals.

One limitation of this study is the different levels of starch and fibre between control and cocoa diet. However, it has been shown that the intake of polyphenols improve the health effects of the intestinal microbiota by activating SCFA excretion, intestinal immune function, and other physiological processes (Kawabata et al., 2019). Therefore, after cocoa intake, the increase in SCFAs is not only due to its fibre content but also to other bioactive compounds mainly polyphenols and theobromine (Martín-Peláez et al., 2017). Further studies will clarify if the effect of cocoa on intestinal microbial populations can be ascribed to cocoa polyphenols and/or theobromine, to dietary fibre or to a possible synergistic activity of all of these dietary components.

In summary, the present study demonstrates for the first time that cocoa supplementation improves intestinal integrity and inflammation in ZDF diabetic rats. Moreover, cocoa intake modifies gut microbiome in ZDF diabetic rats towards a healthier profile and these changes have been closely associated with the improved glucose homeostasis and gut health found in the diabetic animals. Consequently, we suggest that modulation of GM by cocoa may be an important mechanism that could partly mediate beneficial metabolic effects in diabetic animals. Future studies using faecal

519 transplantation from cocoa fed donors could help to address whether or not the alterations in the gut
520 microbiota found in ZDF-Co rats play a pivotal role in mediating the beneficial metabolic effects of
521 cocoa. Likewise, the application of metabolomics to microbiota could provide a more complex analysis
522 to finally advance in the knowledge of the ultimate causality.
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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1.- Effect of cocoa diet in glucose and lipid homeostasis in Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). (A) Food intake. (B) Body weight. (C) Plasma glucose levels. (D) Plasma insulin levels. (E) Glycosylated haemoglobin (HbA1c). (F) Homeostasis model assessment (HOMA)-IR and HOMA-B. (G) Plasma glucose levels during GTT and total area under the curve calculated from the GTT data. (H) Levels of HDL, LDL and TG in serum. Data represent the means ± SD of 6-8 animals. Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 2.- Effects of cocoa diet on colon mucosa of Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). (A) Representative haematoxylin–eosin (H&E) stained sections and crypts depth measured as cells number per hemicrypt of the distal colon mucosa (scale bar 10 μm). (B) Colonic epithelial apoptosis as revealed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (brown-positive nuclei) (scale bar 10 μm) and quantification of apoptotic cells by TUNEL labelling index (%). (C) Representative photographs for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) (brown-positive nuclei) (scale bar 10 μm) and PCNA labelling index (%) in colonic mucosa. (D) Representative images of mucine glycoprotein by PAS staining (magenta) and quantitative analysis of positive PAS staining cells (%) (scale bar 10 μm). (E) Representative IHC photographs of ZO-1 protein expression and the positive area staining (%) (brown-stained) (scale bar 10 μm). Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 3.- Effects of cocoa diet on intestinal inflammation of Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). Representative immunohistochemistry photomicrograph of TNF-α (A), IL-6 (B), MCP-1 (C) and CD45 (D) (brown-stained) in distal colon and inmunoreactive score (scale bar 10 μm). Levels of TNF-α and IL-6 in plasma (E). Values are expressed as mean ± SD (n = 6-8). Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 4.- Bacterial diversity and taxa composition in Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). (A) Number of species identified. (B) Shannon, (C) Simpsons and (D) Chao indexes were measured to evaluate the α-diversity. (E) Unsupervised PCA were carried out to analyse the β-diversity. Each principal component describes most of the variation between samples. (F) Composition of the most abundant bacterial phyla (>0.1%) expressed as a percent of total bacteria and pie graphs of most abundant phyla. (G) *Firmicutes* to *Bacteroidetes* ratio (F/B). Data represent means ± SD of 6–8 animals per condition. Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 5.- Bacterial families composition in Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). (A) Distribution bar-plot of families with relative abundance greater than 1%. (B) Composition of the most abundant families modified with diabetes or with cocoa expressed as a percent of total bacteria. Data represent means ± SD of 6–8 animals per condition. Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 6.- Genera and species composition in Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). (A) Distribution bar-plot of genera with relative abundance greater than 1%. (B) Composition of the most abundant bacterial genera modified with diabetes or with cocoa expressed as a percent of total bacteria. (B) Composition of the most abundant bacterial species modified with diabetes or with cocoa expressed as a percent of total bacteria. Data represent means ± SD of 6–8 animals per condition. Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 7.- SCFA- and lactate-producing bacteria and SCFA and lactate faeces levels in Zucker lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co). (A) Sum of all SCFA- and lactate-producing genera expressed as a percent of total bacteria. (B) Acetate, butyrate, propionate and lactate levels in faeces. (C–F) Most abundant acetate-, butyrate-, propionate- and lactate-producing genera expressed as a percent of total bacteria. Data represent means ± SD of 6–8 animals per condition. Means sharing the same letter are not significantly different from each other ($P<0.05$)

Figure 8.- Correlation analysis between gut microbiota and host parameters in diabetic rats. (A) Heatmap of correlation between the main significantly altered genera in the gut microbiota and host parameters related to diabetes and intestinal integrity and inflammation. Pearson correlation values were used for the matrix. The intensity of the colour represents the degree of association. *Denotes adjusted $P < 0.05$.

784 **Table 1.** Composition of the experimental control and cocoa-rich diets.
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Component (g/Kg dry weight)	Control	Cocoa
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ (*tert*-butylhydroquinone)	0.008	0.008
Mineral mix.	35	35
Vitamin mix.	10	10
L-Cys	1.8	1.8
Cholin bitartrate	2.5	2.5
Cellulose	100	66
Starch	415.7	349.7
Cocoa podwer	-	100
Energy (kJ/kg diet)	15048	15048

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